



Flack, E. K. P., Chidwick, H. S., Guchhait, G., Keenan, T., Budhadev, D., Huang, K., Both, P., Mas Pons, J., Ledru, H., Rui, S., Stafford, G. P., Shaw, J. G., Galan, M. C., Flitsch, S., Thomas, G. H., & Fascione, M. A. (2020). Biocatalytic Transfer of Pseudaminic Acid (Pse5Ac7Ac) Using Promiscuous Sialyltransferases in a Chemoenzymatic Approach to Pse5Ac7Ac-Containing Glycosides. *ACS Catalysis*, 10(17), 9986-9993. <https://doi.org/10.1021/acscatal.0c02189>

Peer reviewed version

Link to published version (if available):  
[10.1021/acscatal.0c02189](https://doi.org/10.1021/acscatal.0c02189)

[Link to publication record in Explore Bristol Research](#)  
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via American Chemical Society at <https://doi.org/10.1021/acscatal.0c02189> . Please refer to any applicable terms of use of the publisher.

## University of Bristol - Explore Bristol Research

### General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:  
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

# Biocatalytic Transfer of Pseudaminic Acid (Pse5Ac7Ac) using Promiscuous Sialyltransferases in a Chemoenzymatic Approach to Pse5Ac7Ac Containing Glycosides

Emily K. P. Flack,<sup>a</sup> Harriet S. Chidwick,<sup>a</sup> Goutam Guchhait,<sup>a</sup> Tessa Keenan,<sup>a</sup> Darshita Budhadev,<sup>a</sup> Kun Huang,<sup>c</sup> Peter Both,<sup>c</sup> Jordi Mas Pons,<sup>d</sup> Helene Ledru,<sup>d</sup> Shengtao Rui,<sup>c</sup> Graham P. Stafford,<sup>f</sup> Jonathan G. Shaw,<sup>c</sup> M. Carmen Galan,<sup>d</sup> Sabine Flitsch,<sup>c</sup> Gavin H. Thomas,<sup>b</sup> and Martin A. Fascione<sup>a\*</sup>

<sup>a</sup>Department of Chemistry, University of York, York, YO10 5DD, UK.

<sup>b</sup>Department of Biology, University of York, York, YO10 5DD, UK.

<sup>c</sup>Manchester Institute of Biotechnology, School of Chemistry, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK

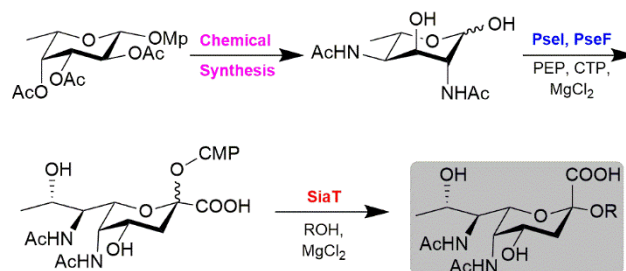
<sup>d</sup>School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK

<sup>e</sup>Department of Infection and Immunity, University of Sheffield, Sheffield, S10 2RX, UK

<sup>f</sup>School of Clinical Dentistry, University of Sheffield, Sheffield, S10 2TA, UK

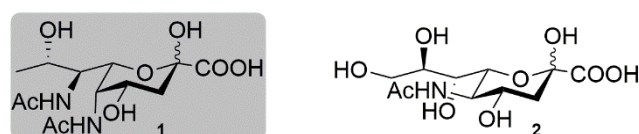
**KEYWORDS:** Chemo-enzymatic synthesis, Pseudaminic acid glycosides, Sialyltransferases, Sialic acid mimics, Biocatalysis

**ABSTRACT:** Pseudaminic acid (Pse5Ac7Ac) is a non-mammalian sugar present on the cell surface of a number of bacteria including *Pseudomonas aeruginosa*, *Campylobacter jejuni* and *Acinetobacter baumannii*. However, the role Pse5Ac7Ac plays in host-pathogen interactions remains underexplored, particularly compared to its ubiquitous sialic acid analogue Neu5Ac. This is primarily due to a lack of access to difficult to prepare Pse5Ac7Ac glycosides. Herein we describe the *in vitro* biocatalytic transfer of an activated Pse5Ac7Ac donor onto glycosyl acceptors enabling the enzymatic synthesis of Pse5Ac7Ac containing glycosides. In a chemoenzymatic approach, chemical synthesis initially afforded access to a late stage Pse5Ac7Ac biosynthetic intermediate, which was subsequently converted to the desired CMP-glycosyl donor in a one-pot-two-enzyme process using biosynthetic enzymes. Finally screening a library of 13 sialyltransferases (SiaT) with the unnatural substrate enabled the identification of a promiscuous inverting SiaT capable of turnover to afford  $\beta$ -Pse5Ac7Ac terminated glycosides.



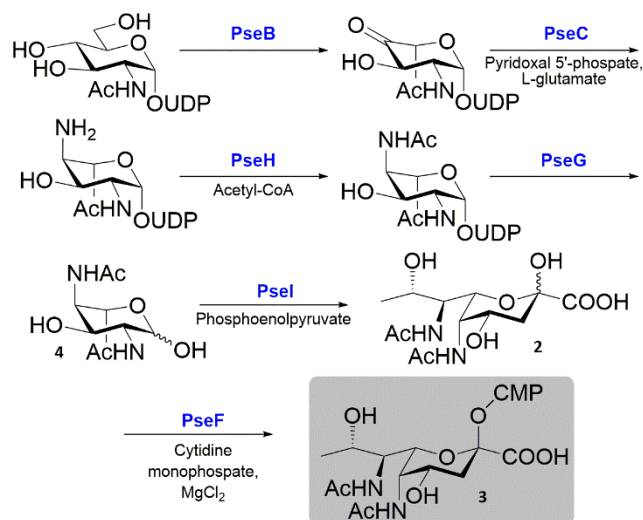
## INTRODUCTION

Nonulosonic acids (NulOs) are nine-carbon  $\alpha$ -keto-acid sugars that are ubiquitous in nature, occurring in several cell surface glycoconjugates where they play a crucial role in cell-cell interactions.<sup>1-4</sup> One nonulosonic acid of particular interest is non-eukaryotic  $\alpha$ -5,7-diacetamido-3,5,7,9-tetra-deoxy-L-glycero-L-manno-non-2-ulosonic acid, or pseudaminic acid (Pse5Ac7Ac **1**), and its derivatives.<sup>5</sup> Upon its discovery within the lipopolysaccharide *O*-antigen of *Pseudomonas aeruginosa* and *Shigella boydii* in the mid-1980s,<sup>6</sup> it was noted that Pse5Ac7Ac **1** possesses structural similarities to the widely prevalent *N*-acetyl neuraminic acid (Neu5Ac)<sup>7</sup> **2** (Figure 1, which has D-glycero-D-galacto stereochemistry).<sup>8</sup> A number of derivatives differentially substituted at the N5 or N7 positions of the Pse backbone have subsequently been identified in a range of glycoconjugates attached through both axial and equatorial glycosidic linkages,<sup>9-11</sup> in several multidrug resistant pathogens<sup>5</sup> where they play a crucial role in virulence.<sup>5,12-14</sup>



**Figure 1** Nonulosonic acid structures; the pseudaminic acid Pse5Ac7Ac **1**, and sialic acid Neu5Ac **2**.

Neu5Ac **2** often exists as the terminal sugar on human cell surface glycoconjugates, and is therefore an accessible binding site for invaders, such as the influenza virus,<sup>15</sup> but also for interactions with host proteins, specifically Siglecs which are lectins primarily present on host immune cells.<sup>16</sup> Siglecs play an integral role in immune tolerance and help the immune system to recognise Neu5Ac and interpret its presence on the cell as a sign of “self”,<sup>17</sup> a mechanism which can be exploited by bacteria able to camouflage themselves in surface Neu5Ac and evade immune detection in an example of “molecular mimicry”.<sup>18</sup> Despite its structural similarity to Neu5Ac **2** and



**Scheme 1** Biosynthesis of CMP-Pse5Ac7Ac **3** via the key hexose intermediate 6-deoxy-L-AldiNAc **4**.

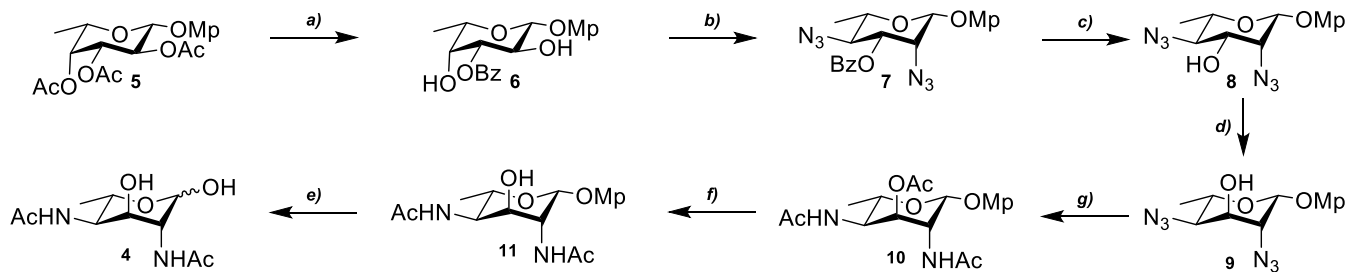
forementioned presence on pathogenic bacterial cell surfaces, there are precious few studies<sup>19</sup> exploring how Pse5Ac7Ac **1** might bind to host proteins compared to Neu5Ac **2**, and therefore if bacteria employ Pse glycans to aid immune evasion. Such studies have been hindered by a lack of access to Pse-glycosides, specifically those which mimic natural Neu5Ac capped glycoconjugates and would provide a means to compare the effects of a terminal Neu5Ac or Pse5Ac7Ac architecture on glycan-receptor interactions. This is because production of Pse glycosides by purely chemical methods is rare, and although impressively elegant, all reported strategies to date have been multi-step resulting in a low overall yield, and face additional challenges in controlling the stereochemistry of Pse-glycosidic linkages during glycosylation.<sup>20–27</sup> Conversely, the highly stereo- and regioselective nature of enzymatic glycosylation make it an attractive route to Pse-glycosides. *In vivo* studies have tentatively identified glycosyltransferases proposed to utilise CMP-Pse donors (pseudaminyltransferases) in a range of bacteria.<sup>28, 29</sup> However, currently no direct *in vitro* enzymatic transfer of CMP-Pse using a glycosyltransferase has been reported. Furthermore the sole *in vitro* enzymatic synthesis of CMP-Pse5Ac7Ac **3** in the literature is prohibitively expensive requiring both costly UDP-GlcNAc starting material and an acetyl-CoA cofactor in the early steps in the pathway (Scheme 1).<sup>30</sup>

Therefore to facilitate future comparisons of the effects of

Neu5Ac and Pse5Ac7Ac capped glycoconjugates on protein binding interactions, herein we address the lack of practical procedures to Pse-glycosides through the union of both chemical and enzymatic synthesis. Concluding with the transfer of CMP-Pse5Ac7Ac **3** onto a range of glycosyl acceptors exploiting a promiscuous sialyltransferase to afford  $\beta$ -2,3/2,6-linked Pse di- and trisaccharides mimics. We initially targeted the chemical synthesis of 6-deoxy-L-AldiNAc **4** (Scheme 1), a biosynthetic precursor of Pse5Ac7Ac **1**, and a substrate for PseI, a Pse5Ac7Ac synthase.<sup>12, 31</sup> Our aim was to utilise PseI in combination with PseF, a CMP-Pse5Ac7Ac acid synthetase,<sup>30</sup> in a one-pot procedure to synthesise CMP-Pse5Ac7Ac **3** from the reducing sugar **4**. The CMP-sugar **3** could then be used as a potential glycosyl donor in a sialyltransferase (SiaTs) activity screen, with the aim of identifying promiscuous enzymes capable of synthesising Pse containing glycosides.

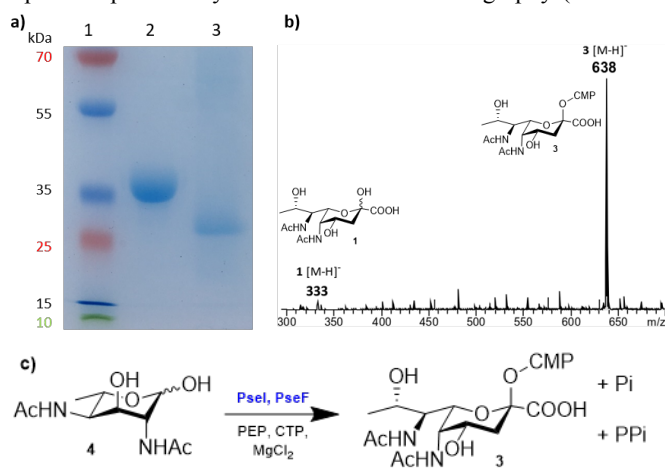
## RESULTS AND DISCUSSION

**Chemical synthesis of 6-deoxy-L-AldiNAc 4.** We established an efficient chemical route to 6-deoxy-L-AldiNAc **4** starting from the readily synthesised L-fucoside **5** (Scheme 2).<sup>32</sup> The synthesis required a triple inversion of stereochemistry at the 2, 3, and 4 positions, with the order in which these inversions were performed proving crucial to success. Following global deacetylation of **5**, regioselective *O*-3 benzylation was achieved with diphenyl borinate to afford the L-fucosyl 2,4-diol **6**. Treatment of **6** with  $\text{TiF}_2\text{O}$  in pyridine yielded a 2,4-bis-triflate, which was then treated with  $\text{NaN}_3$  to access the rare deoxy-amino-L-mannose derivative **7** via double, parallel inversions.<sup>33</sup> Notably when double inversions were attempted on the *O*-3 benzoyl epimer of **6**, only decomposition was observed, presumably due to the axial benzoyl group increasing steric hindrance during equatorial attack of *O*-4, as previously also noted by Ito and co-workers.<sup>24</sup> Debenzylation afforded compound **8**, which was then triflylated at *O*-3 to produce an intermediate triflate, and once more subjected to inversion without purification through treatment with  $\text{TBANO}_2$ . The reaction proceeded cleanly and 2,4-di-azido altopryronaside **9** was isolated in 46% yield, over two steps. However, we noted that if the  $\alpha$ -anomer of **8** was treated under similar conditions, or subjected to oxidation and subsequent hydride reduction, no inversion of stereochemistry at *O*-3 was observed in our hands, likely a result of unfavourable 1,3-diaxial interaction experienced during axial attack upon *O*-3. Staudinger reduction of the 2,4-di-azido altopryronaside **9** with  $\text{PMe}_3$ , followed by acetylation, afforded compound **10**, which was then selec-



tively *O*-deacetylated to afford 2,4-di-acetamido altropyronaside **11**. Ceric ammonium nitrate assisted deprotection of the anomeric methoxyphenyl group finally yielded the desired 6-deoxy-AltDiNAc reducing sugar **4** in an 11% overall yield.

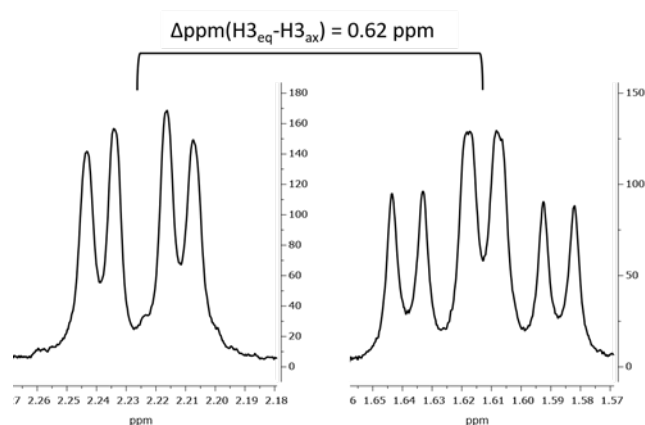
**Enzymatic production of CMP-Pse5Ac7Ac **3**.** Synthesis of the desired CMP-Pse5Ac7Ac **3** nucleotide donor was achieved utilizing the final two enzymes from the biosynthetic pathway; PseI and PseF (Figure 2). *C. jejuni* PseI was initially expressed and purified under optimized conditions affording 20 mg L<sup>-1</sup> (Figure 2a).<sup>31</sup> Sequence alignment with *H. pylori* PseF<sup>34</sup> revealed a putative CMP-Pse5Ac7Ac synthetase gene present in the gram-negative *Aeromonas caviae* genome.<sup>28</sup> Subsequent expression of *A. caviae* PseF was optimised and resulted in the purification of soluble enzyme affording 13 mg L<sup>-1</sup> (Figure 2a). Activity of the Pse5Ac7Ac synthase PseI was monitored by negative ion electrospray ionization (ESI) LC-MS during incubation with 6-deoxy-L-AltDiNAc **4** at pH 7.4 with a small excess of the required phosphoenol pyruvate co-factor.<sup>31</sup> A peak associated with production of Pse5Ac7Ac **1** ([M-H]<sup>-</sup> 333) was immediately observed without addition of exogenous divalent metal ions (Figure S2). Upon addition of *A. caviae* PseF to the reaction mixture with excess CTP and MgCl<sub>2</sub> a peak associated with the production of CMP-Pse5Ac7Ac **3** ([M-H]<sup>-</sup> 638) was observed. Hence demonstrating that CMP-Pse5Ac7Ac **3** can be enzymatically produced in one-pot from chemically synthesized 6-deoxy-L-AltDiNAc **4** (Figure 2c). The reaction was stopped after four hours and the product purified by size exclusion chromatography (Bio-Gel®



**Figure 2** a) 12% SDS PAGE analysis of purified enzymes; Lane 1: Molecular weight ruler, Lane 2: *C. jejuni* PseI (40.8 kDa), Lane 3: *A. caviae* PseF (28.0 kDa) b) Negative ESI LC-MS for the one-pot, two enzyme synthesis of CMP-Pse5Ac7Ac **3** from 6-deoxy-AltDiNAc **4** c) Use of the chemically derived 6-deoxy-L-AltDiNAc **4** in the one-pot enzymatic synthesis of CMP-Pse5Ac7Ac **3**.

P-2 resin Biorad), NMR and LC-MS characterization of the enzymatic product by comparison to previously published data confirmed the synthesis of  $\alpha$ CMP-Pse5Ac7Ac **3** (Figure S25-

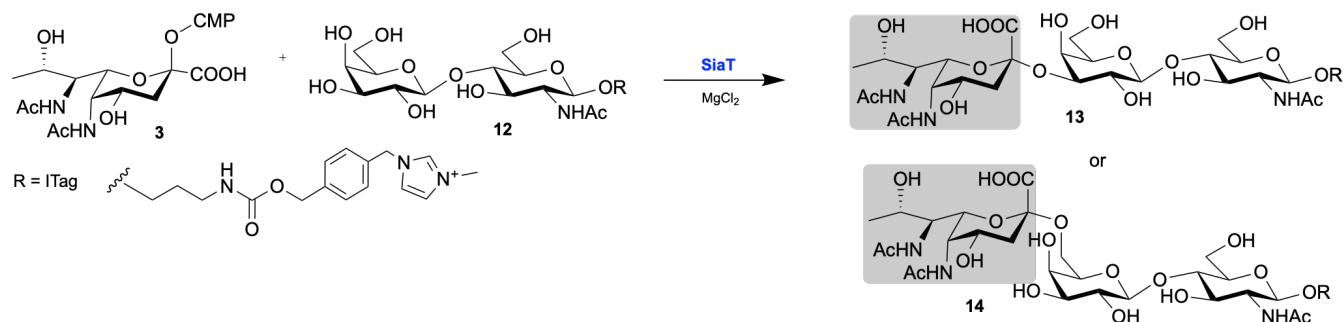
26).<sup>30</sup> Calculation of the difference in chemical shift between the H3<sub>ax</sub> and H3<sub>eq</sub> ( $\Delta$ ppm H3<sub>eq</sub>-H3<sub>ax</sub> = 0.62 ppm) was indicative of the axially orientated *O*-CMP group (Figure 3).<sup>35,36</sup>



**Figure 3** The chemical shift of the H3eq (2.23 ppm) and H3ax (1.61 ppm) proton NMR peaks used to assign the PseF enzymatic product as  $\alpha$ CMP-Pse5Ac7Ac **3**.

**Sialyltransferase activity screen using  $\alpha$ -CMP-Pse5Ac7Ac **3**.** *In vitro* studies of well-characterised sialyltransferases (SiaTs) have shown that a number of enzymes have promiscuity towards both the donors and acceptors they can turnover. A range of natural and bioorthogonal tagged CMP-Neu5Ac derivatives and other CMP-nonulosonic acids (such as CMP-Leg5Ac7Ac) have previously been utilised as donors.<sup>6, 35, 37-38</sup> Pse5Ac7Ac **1** differs from Neu5Ac **2** functionally; C7 has an acetamido and C9 loses a hydroxyl, and stereochemically; C5, C7 and C8 are epimeric. However SiaT promiscuity had previously been observed with sugars that include some of these modifications, C9 deoxy<sup>37</sup> and C7 *N*-acetyl<sup>38</sup> sugars for example, therefore it was reasoned that these enzymes may also be functional in the chemoenzymatic synthesis of Pse5Ac7Ac-based glycosides. A library of 13 bacterial SiaTs were therefore screened for their ability to utilise  $\alpha$ CMP-Pse5Ac7Ac **3** as a glycosyl donor, including the three enzymes that had previously shown activity towards CMP-Leg5Ac7Ac.<sup>37, 39</sup> A range of characterised and putatively assigned SiaTs were considered (Table S1), from three different GT families; GT42, GT52 and GT80, with an imidazolium tagged-*N*-acetyllactosamine (LacNAc-ITag)<sup>40</sup> **12** acceptor (Scheme 3). Two SiaTs chosen had previously been shown to catalyse the glycosylation of CMP-Neu5Ac and a LacNAc derivative labelled with a *N*-benzenesulfonyl-type ITag,<sup>41</sup> which bears a similar imidazolium label to LacNAc-ITag **12**. The use of the cationic ITag label decreases the mass spectrometry detection limit for observing reaction components and increases the spectral peak intensity of LacNAc-ITag containing moieties, thus facilitating the screening of enzyme activity.<sup>42-44</sup>





**Scheme 3** SiaT catalyzed transfer of CMP-Pse5Ac7Ac **3** onto LacNAc-ITag **12**.

Reaction conditions were identical for all SiaTs; incubating a 1:4 ratio of donor to acceptor at pH 7.5, for 18 hours. Analysis of ITag containing compounds with MALDI-ToF showed starting material remained in all reactions but an additional peak was present with four enzymes (all GT80), which was assigned to the desired Pse5Ac7Ac-LacNAc-ITag (*m/z* 985.4) (Table S1, Figure S3). Based on the reported enzymatic characterisation of these enzymes, under these reaction conditions it was predicted that the *Photobacterium* sp. *JT-ISH-224*<sup>45</sup>, *Pasteurella dagmatis*<sup>46</sup> and truncated *Pasteurella multocida*<sup>38</sup> SiaTs would all catalyse production of the  $\beta$ -2,3-linked glycoside **13** and the *Photobacterium leiognathi*<sup>47</sup> SiaT would result in a  $\beta$ -2,6-linked glycoside **14**. Not all SiaTs tested herein have previously been characterised and hence negative hits are to be treated with caution as pseudo-pseudaminyltransferase activity may also be possible under alternate conditions.<sup>38</sup>

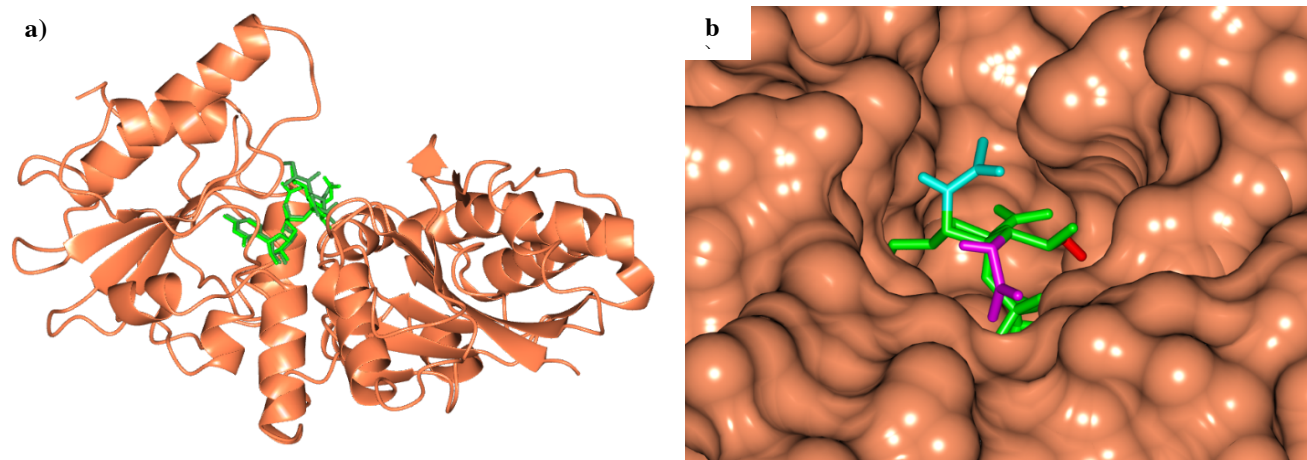
To corroborate the MALDI-TOF results for the positive hits, the same reactions were analysed using negative ESI LC-MS (Figure S4), and in all cases no remaining CMP-Pse5Ac7Ac **3** starting material was detected. Considering the *Pasteurella multocida* SiaT (tPm0188Ph) affords  $\beta$ -2,3-linked glycosides at pH 7.5, but can also potentially be tuned to produce  $\alpha$ -2-6-glycosides, under different conditions<sup>38</sup> it was chosen for further investigation. Specifically,  $\alpha$ CMP-Pse5Ac7Ac **3** was screened with a range of galactose acceptors **15–20**, in a 1:4 donor to acceptor ratio (Table 1) to profile tPm0188Ph acceptor promiscuity. Negative ESI UPLC-MS standard curves for Pse5Ac7Ac **1** and  $\alpha$ CMP-Pse5Ac7Ac **3** at known concentrations (Figure S5) were used to calculate the level of conversion to the glycoside product by determining the amount of unreacted material present. In 4 h experiments high levels of conversion (>75%) were achieved with five of the galactose-based acceptors **15**, **17–20** (Table 1). Similar to results previously obtained with the CMP-Neu5Ac donor, LacNAc-ITag **12** was also accepted particularly well by this enzyme (90% and 91% conversion respectively),<sup>38</sup> as was Gal $\beta$ -pNP **20** (91% conversion). This was significantly higher than with monosaccharides **15** and **17**, presumably as **18–20** more closely mimic the structure of the native disaccharide acceptor.<sup>48</sup> A 57% conversion was also observed with the unnatural 6F-galactose **16** demonstrating that under the reaction conditions screened the *P. multocida* enzyme is indeed likely forming a 2,3-linkage. It was proposed that the reduction of activity with 6F-galactose **16** could be attributed to a loss of hydrogen bonding to Asn85 that is predicted in the galactose binding region with the native C6-OH.<sup>48</sup> A time-course experiment using LacNAc acceptor **19** demonstrated that the majority of the  $\alpha$ CMP-Pse5Ac7Ac **3** donor was turned over to glycoside product within 1 h (Figure S10), and although some formation of hydrolysis product Pse5Ac7Ac **1** was observed by negative ion LCMS, the relative proportion did not change over the course of the reaction (0–4 h) suggesting minimal enzymatic hydrolysis of the donor.

Docking<sup>49</sup> of  $\alpha$ CMP-Pse5Ac7Ac **3** into the *P. multocida* crystal structure<sup>48</sup> showed that this donor could be accepted in a conformation analogous to that reported for bound CMP-3F(a)Neu5Ac (Figure 4.a) reinforcing the donor promiscuity observed experimentally. Previous donor-bound crystal structures highlight that the majority of interactions occur with the nucleotide moiety which is buried within the enzyme active site,<sup>48</sup> docking of  $\alpha$ CMP-Pse5Ac7Ac **3** does not disturb any of these interactions, despite a slight perturbation of the phosphate group (Figure S7). Importantly when  $\alpha$ CMP-Pse5Ac7Ac **3** is docked in its lowest energy conformation with the CMP moiety occupying this site, there is no steric clashes between the Pse5Ac7Ac sugar moiety and the enzyme (Figure 4.b). Similarly, the binding affinity of docked  $\alpha$ CMP-Pse5Ac7Ac **3** (-10 kcal mol<sup>-1</sup>) was only marginally lower than docked  $\beta$ CMP-Neu5Ac (-10.2 kcal mol<sup>-1</sup>) suggesting that any loss of binding interactions due to structural differences are compensated for by other interactions. However, it is notable that in a direct competition experiment between the  $\beta$ CMP-Neu5Ac and  $\alpha$ CMP-Pse5Ac7Ac **3** donors in the presence of LacNAc acceptor **19** formation of 10-fold greater Neu5Ac-LacNAc over Pse5Ac7Ac-LacNAc was observed (Figure S8), indicating that *in vitro* the *P. multocida* enzyme has an established preference for the Neu5Ac scaffold.

Co-crystallisation with CMP-3F(a)Neu5Ac previously identified five hydrogen bonds (two water-mediated) and one ion pair interaction between the Neu5Ac moiety and enzyme.<sup>48</sup> Analysis of  $\alpha$ CMP-Pse5Ac7Ac docked into tPm0188 shows that there are also five hydrogen bonds (one water-mediated) and one ion pair interaction possible, albeit to different resi-

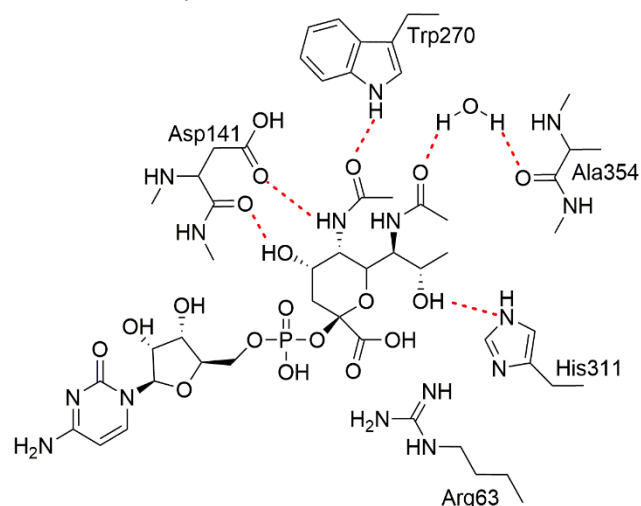
Acceptor	Conversion	
<b>15</b>	78%	
<b>16</b>	57%	
<b>17</b>	80%	
<b>18</b>	90%	
<b>19</b>	91%	
<b>20</b>	91%	

Co-crystallisation with CMP-3F(a)Neu5Ac previously identified five hydrogen bonds (two water-mediated) and one ion pair interaction between the Neu5Ac moiety and enzyme.<sup>48</sup> Analysis of  $\alpha$ CMP-Pse5Ac7Ac docked into tPm0188 shows that there are also five hydrogen bonds (one water-mediated) and one ion pair interaction possible, albeit to different resi-



**Figure 4**  $\alpha$ CMP-Pse5Ac7Ac **3** (green cylinder model) docked into *Pasteurella multocida* tPm0188Ph (coral) **a**) overlaid with CMP-Neu5Ac (lawn green cylinder model) to show the heavily conserved binding motif, and **b**) a tPm0188Ph space filling model (coral) to show the orientation of the epimeric Pse5Ac7Ac sugar ring substituents (C5 acetamido-cyan, C7 acetamido-magenta, C8 hydroxyl-red).

dues in the active site in some cases (Figure 5). The C2 carboxyl is orientated to allow for ion pair interactions with Arg63 in both Neu5Ac and Pse5Ac7Ac, similarly the C4 hydroxyl in both molecules can form an H-bond with the Asp141 carbonyl backbone. The Trp270 side chain forms a H bond with both Neu5Ac and Pse5Ac7Ac moieties, the C7 hydroxyl and C5 acetamido carbonyl respectively, which occupy a similar space due to the opposing stereochemistry at these two centres. In Pse5Ac7Ac, the C5 acetamido group forms a second H bond with the amine proton interacting with the Asp141 side chain carbonyl in this configuration, whereas this H bond is to Ser143 in Neu5Ac. The C8 hydroxyl in both molecules form H bonds, a water mediated bond occurs with Thr267 in Neu5Ac, however in Pse5Ac7Ac the C8 hydroxyl can directly interact with His311 due being epimerised at this position. Finally, the Pse5Ac7Ac C7 acetamido group has a water mediated H bond between the carbonyl moiety and the backbone carbonyl of Ala354.



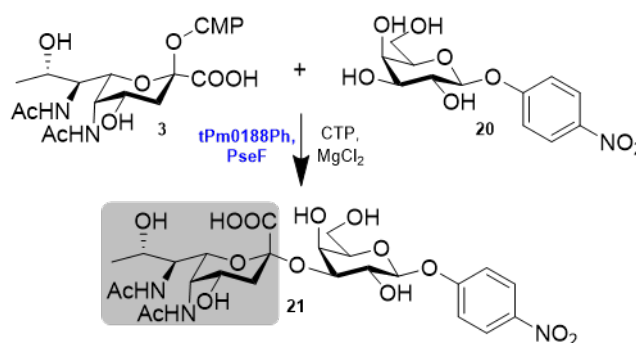
**Figure 5** Binding interactions between the Pse5Ac7Ac moiety and *Pasteurella multocida* tPm0188Ph when  $\alpha$ CMP-Pse5Ac7Ac **3** is docked *in silico* into the active site.

**tPm0188Ph catalyzed synthesis of Pse5Ac7Ac $\beta$ -2,3-Gal $\beta$ -pNP **21**.** The *P. multocida* enzyme (tPm0188Ph) has previously been shown to act as an inverting SiaT transferring  $\beta$ CMP-

Neu5Ac to galactosides to preferentially make an  $\alpha$ -2,3-linkage at pH 7.5-9.0 or an  $\alpha$ -2,6-linkage at pH 5.0-5.5.<sup>38</sup> Therefore a  $\beta$ -2,3-Pse5Ac7Ac-galactoside would be the predicted product when using tPm0188Ph with  $\alpha$ CMP-Pse5Ac7Ac **3** under the reaction conditions employed (pH 7.5) (Scheme 4). However further characterisation of the pseudaminy galactoside was required to confirm the regio- and stereo- selectivity of the pseudo-pseudaminy transferase activity. Therefore a preparative scale reaction containing  $\alpha$ CMP-Pse5Ac7Ac **3** (9.5 mg), four equivalents of Gal $\beta$ -pNP **20** (20.5 mg) and tPm0188Ph was used to afford enough product for purification and characterization (see SI for full details). PseF, CTP and MgCl<sub>2</sub> were also included in the reaction mixture to recycle any hydrolysed Pse5Ac7Ac **1** into  $\alpha$ CMP-Pse5Ac7Ac **3** and drive the reaction towards the product. The reaction was stopped when after 18 hours (conversion > 90%) and following purification, NMR analysis confirmed the product as  $\beta$ -2,3-Pse5Ac7Ac-Gal $\beta$ -pNP **21** (Figure S27-28), formed in a 2.1 mg (23 %) yield. <sup>13</sup>C NMR comparison of the Gal $\beta$ -pNP **20** and product **21** revealed a downfield chemical shift of the galactose C3 by 2.65 ppm, with no significant galactose C6 chemical shift perturbation, indicative of glycosylation at the galactose C3.<sup>38</sup> Once again, the anomeric stereochemistry was assigned using the difference in chemical shift between the H3<sub>ax</sub> and H3<sub>eq</sub> with a value of  $\Delta$ ppm H3<sub>eq</sub>-H3<sub>ax</sub> = 0.9 ppm indicative and characteristic of a Pse5Ac7Ac  $\beta$ -linkage (Figure 6).<sup>35,36</sup>

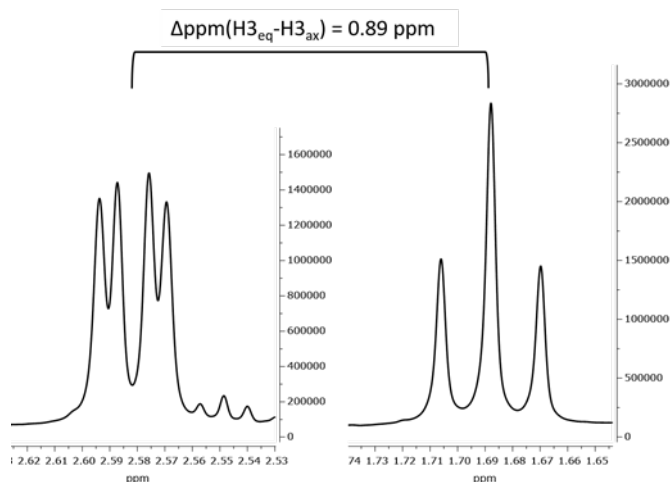
## CONCLUSIONS

This work provides the first examples of chemoenzymatically synthesised Pse containing glycosides, which are in in-



**Scheme 4** tPm0188Ph catalysed synthesis of Pse5Ac7Ac $\beta$ -2,3-Gal $\beta$ -pNP **21**.

creasingly high demand for biological studies. Given that the chemical syntheses of such molecules are particularly arduous, the use of enzymes in tandem with synthetic chemistry affords



**Figure 6** The chemical shift of the H3eq (2.58 ppm) and H3ax (1.69 ppm) proton NMR peaks used to assign the *P. multocida* tPm0188Ph SiaT product as Pse5Ac7Ac $\beta$ -2,3-Gal $\beta$ -pNP **21**.

a more expedient and efficient route to the synthesis of this important class of glycans. This work also provides Pse5Ac7Ac containing mimics of Neu5Ac capped glycoconjugates, enabling comparative exploration of receptor-glycan interactions, particularly relevant for probing the interactions between bacterial surfaces and Siglec proteins integral to host immune responses. Notably, as no native pseudaminyltransferases have yet been shown to be active *in vitro*, our demonstration that the well-characterised, highly stable, sialyltransferase from *P. multocida* displays activity with  $\alpha$ -CMP-Pse5Ac7Ac **3**, provides an impetus to study the potential turnover of Pse glycans by other sialic acid processing enzymes. Furthermore *P. multocida* tPm0188Ph and other promiscuous sialyltransferases may offer an expedient route to the synthesis of galactose linked Pse-glycosides present in the *O*-antigens of

1. Varki, A., Diversity in the Sialic Acids. *Glycobiology* **1992**, *2*, 25-40.
2. Knirel, Y. A.; Shashkov, A. S.; Tsvetkov, Y. E.; Jansson, P.-E.; Zähringer, U., 5,7-Diamino-3,5,7,9-Tetradeoxynon-2-Ulosonic Acids in Bacterial Glycopolymers: Chemistry and Biochemistry. In *Adv. Carbohydr. Chem. Biochem.*, Academic Press: 2003; Vol. 58, pp 371-417.
3. Angata, T.; Varki, A., Chemical Diversity in the Sialic Acids and Related  $\alpha$ -Keto Acids: An Evolutionary Perspective. *Chem. Rev.* **2002**, *102*, 439-470.
4. Chen, X.; Varki, A., Advances in the Biology and Chemistry of Sialic Acids. *ACS Chem. Biol.* **2010**, *5*, 163-176.
5. Zunk, M.; Kiefel, M. J., The Occurrence and Biological Significance of the  $\alpha$ -Keto-Sugars Pseudaminic Acid and Legionaminic Acid

pathogenic bacteria including enteroinvasive *E. coli*,<sup>50</sup> and *S. boydii*<sup>51</sup> serotypes.

## ASSOCIATED CONTENT

### Supporting Information.

The supporting Information, including full experimental, assay results and NMR characterization, is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

\* Dr Martin Fascione, Email: [martin.fascione@york.ac.uk](mailto:martin.fascione@york.ac.uk)

### Author Contributions

E.K.P.F., H.S.C., T.K., K.H., P.B. performed protein production and enzymatic transformations; G.G., D.B., J.M.P., and H.L. performed chemical transformations; S.R. cloned A. caviae PseF; G.P.S., J.G.S., M.C.G., S.F., G.T., and M.A.F. supervised the project; M.A.F., E.K.P.F., and H.S.C., wrote the paper and designed the study, and all authors commented on the paper.

### Funding Sources

This work was supported by The University of York, the BBSRC (BB/M02487X/1, T.K.), the EPSRC (EP/P030653/1, D.B.), and The Rosetrees Trust. MCG thank the European Research Council (ERC-COG: 648239) and BBSRC (BB/M028976/1, J.M.P and H.L.).

### Notes

There are no conflicts of interest to declare.

## ACKNOWLEDGEMENTS

We thank Dr Simon Charnock of Proxomix Ltd for supplying SiaTs used in the pseudaminyltransferase activity screen, Dr. Ed Bergstrom and The York Centre of Excellence in Mass Spectrometry. The York Centre of Excellence in Mass Spectrometry was created thanks to a major capital investment through Science City York, supported by Yorkshire Forward with funds from the Northern Way Initiative, and subsequent support from EPSRC (EP/K039660/1; EP/M028127/1).

## REFERENCES

- within Pathogenic Bacteria. *RSC Adv.* **2014**, *4*, 3413-3421.
6. Knirel, Y. A.; Vinogradov, E. V.; L'vov, V. L.; Kocharova, N. A.; Shashkov, A. S.; Dmitriev, B. A.; Kochetkov, N. K., Sialic Acids of a New Type from the Lipopolysaccharides of *Pseudomonas Aeruginosa* and *Shigella Boydii*. *Carbohydr. Res.* **1984**, *133*, C5-C8.
7. Traving, C.; Schauer, R., Structure, Function and Metabolism of Sialic Acids. *Cell. Mol. Life. Sci.* **1998**, *54*, 1330-1349.
8. Varki A, C. R., Esko Jd, Freeze Hh, Stanley P, Bertozzi Cr, Hart Gw, Etzler Me. , *Essentials of Glycobiology*. Second ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 2009.
9. Knirel, Y. A.; Kocharova, N. A.; Shashkov, A. S.; Dmitriev, B. A.; Kochetkov, N. K.; Stanislavskii, E. S.; Mashilova, G. M., Somatic Antigens of *Pseudomonas Aeruginosa*. The Structure of *O*-Specific Polysaccharide

- Chains of the Lipopolysaccharides from *P. Aeruginosa* O5 (Lanyi) and Immunotype 6 (Fisher). *Eur. J. Biochem.* **1987**, *163*, 639-52.
10. Staaf, M.; Weintraub, A.; Widmalm, G., Structure Determination of the O-Antigenic Polysaccharide from the Enteroinvasive *Escherichia Coli* O136. *Eur. J. Biochem.* **1999**, *263*, 656-661.
  11. Gil-Serrano, A. M.; Rodríguez-Carvajal, M. A.; Tejero-Mateo, P.; Espartero, J. L.; Menendez, M.; Corzo, J.; Ruiz-Sainz, J. E.; Buendía-Clavería, A. M., Structural Determination of a 5-Acetamido-3,5,7,9-Tetradexy-7-(3-Hydroxybutyramido)-L-Glycero-L-Manno-Nonulosonic Acid-Containing Homopolysaccharide Isolated from *Sinorhizobium Fredii* Hh103. *Biochem. J.* **1999**, *342*, 527-535.
  12. Schirm, M.; Soo, E. C.; Aubry, A. J.; Austin, J.; Thibault, P.; Logan, S. M., Structural, Genetic and Functional Characterization of the Flagellin Glycosylation Process in *Helicobacter Pylori*. *Mol. Microbiol.* **2003**, *48*, 1579-1592.
  13. Hopf, P. S.; Ford, R. S.; Zebian, N.; Merckx-Jacques, A.; Vijayakumar, S.; Ratnayake, D.; Hayworth, J.; Creuzenet, C., Protein Glycosylation in *Helicobacter Pylori*: Beyond the Flagellins? *PLoS One* **2011**, *6*, e25722.
  14. Ewing, C. P.; Andreishcheva, E.; Guerry, P., Functional Characterization of Flagellin Glycosylation in *Campylobacter Jejuni* 81-176. *J. Bacteriol.* **2009**, *191*, 7086-7093.
  15. Stencel-Baerenwald, J. E.; Reiss, K.; Reiter, D. M.; Stehle, T.; Dermody, T. S., The Sweet Spot: Defining Virus-Sialic Acid Interactions. *Nat. Rev. Microbiol.* **2014**, *12*, 739-749.
  16. Crocker, P. R.; Paulson, J. C.; Varki, A., Siglecs and Their Roles in the Immune System. *Nat. Rev. Immunol.* **2007**, *7*, 255-266.
  17. Lübbers, J.; Rodríguez, E.; Van Kooyk, Y., Modulation of Immune Tolerance Via Siglec-Sialic Acid Interactions. *Front. Immunol.* **2018**, *9*.
  18. Varki, A.; Gagneux, P., Multifarious Roles of Sialic Acids in Immunity. *Ann. N. Y. Acad. Sci.* **2012**, *1253*, 16-36.
  19. Stephenson, H. N.; Jones, H.; Milioris, E.; Copland, A.; Bajaj-Elliott, M.; Mills, D. C.; Dorrell, N.; Wren, B. W.; Crocker, P. R.; Escors, D., Pseudaminic Acid on *Campylobacter Jejuni* Flagella Modulates Dendritic Cell IL-10 Expression Via Siglec-10 Receptor: A Novel Flagellin-Host Interaction. *J. Infect. Dis.* **2014**, *210*, 1487-98.
  20. Liu, H.; Zhang, Y.; Wei, R.; Andolina, G.; Li, X., Total Synthesis of *Pseudomonas Aeruginosa* 1244 Pilin Glycan Via De Novo Synthesis of Pseudaminic Acid. *J. Am. Chem. Soc.* **2017**, *139*, 13420-13428.
  21. Tsvetkov, Y. E.; Shashkov, A. S.; Knirel, Y. A.; Zähringer, U., Synthesis and Identification in Bacterial Lipopolysaccharides of 5,7-Diacetamido-3,5,7,9-Tetradexy-D-Glycero-D-Galacto- and -D-Glycero-D-Talo-Non-2-Ulosonic Acids. *Carbohydr. Res.* **2001**, *331*, 233-237.
  22. Zunk, M.; Williams, J.; Carter, J.; Kiefel, M. J., A New Approach Towards the Synthesis of Pseudaminic Acid Analogues. *Org. Biomol. Chem.* **2014**, *12*, 2918-2925.
  23. Williams, J. T.; Corcilus, L.; Kiefel, M. J.; Payne, R. J., Total Synthesis of Native 5,7-Diacetylpsudaminic Acid from N-Acetylneuraminic Acid. *J. Org. Chem.* **2016**, *81*, 2607-2611.
  24. Lee, Y. J.; Kubota, A.; Ishiwata, A.; Ito, Y., Synthesis of Pseudaminic Acid, a Unique Nonulopyranoside Derived from Pathogenic Bacteria through 6-Deoxy-Altdinac. *Tetrahedron Lett.* **2011**, *52*, 418-421.
  25. Dhakal, B.; Crich, D., Synthesis and Stereocontrolled Equatorially Selective Glycosylation Reactions of a Pseudaminic Acid Donor: Importance of the Side-Chain Conformation and Regioselective Reduction of Azide Protecting Groups. *J. Am. Chem. Soc.* **2018**, *140*, 15008-15015.
  26. Dhakal, B.; Buda, S.; Crich, D., Stereoselective Synthesis of 5-Epi-A-Sialosides Related to the Pseudaminic Acid Glycosides. Reassessment of the Stereoselectivity of the 5-Azido-5-Deacetamidodialyl Thioglycosides and Use of Triflate as Nucleophile in the Zbiral Deamination of Sialic Acids. *J. Org. Chem.* **2016**, *81*, 10617-10630.
  27. Flack, E. K. P.; Chidwick, H. S.; Best, M.; Thomas, G. H.; Fascione, M. A., Synthetic Approaches for Accessing Pseudaminic Acid (Pse) Bacterial Glycans. *ChemBioChem*. doi:[10.1002/cbic.202000019](https://doi.org/10.1002/cbic.202000019)
  28. Tabei, S. M. B.; Hitchen, P. G.; Day-Williams, M. J.; Merino, S.; Vart, R.; Pang, P.-C.; Horsburgh, G. J.; Viches, S.; Wilhelms, M.; Tomas, J. M.; Dell, A.; Shaw, J. G., An *Aeromonas Caviae* Genomic Island Is Required for Both O-Antigen Lipopolysaccharide Biosynthesis and Flagellin Glycosylation. *J. Bacteriol.* **2009**, *191*, 2851-2863.
  29. Parker, J. L.; Day-Williams, M. J.; Tomas, J. M.; Stafford, G. P.; Shaw, J. G.,



- Identification of a Putative Glycosyltransferase Responsible for the Transfer of Pseudaminic Acid onto the Polar Flagellin of *Aeromonas Caviae* Sch3n. *MicrobiologyOpen* **2012**, *1*, 149-160.
30. Schoenhofen, I. C.; McNally, D. J.; Brisson, J.-R.; Logan, S. M., Elucidation of the Cmp-Pseudaminic Acid Pathway in *Helicobacter Pylori*: Synthesis from Udp-N-Acetylglucosamine by a Single Enzymatic Reaction. *Glycobiology* **2006**, *16*, 8C-14C.
31. Chou, W. K.; Dick, S.; Wakarchuk, W. W.; Tanner, M. E., Identification and Characterization of Neub3 from *Campylobacter Jejuni* as a Pseudaminic Acid Synthase. *J. Biol. Chem.* **2005**, *280*, 35922-35928.
32. Mandal, P. K., Convergent Synthesis of the Pentasaccharide Repeating Unit of the O-Antigen of *Escherichia Coli* O36. *Synthesis* **2015**, *47*, 836-844.
33. Sanapala, S. R.; Kulkarni, S. S., Expedient Route to Access Rare Deoxy Amino L-Sugar Building Blocks for the Assembly of Bacterial Glycoconjugates. *J. Am. Chem. Soc.* **2016**, *138*, 4938-4947.
34. Guerry, P.; Ewing, C. P.; Schirm, M.; Lorenzo, M.; Kelly, J.; Pattarini, D.; Majam, G.; Thibault, P.; Logan, S., Changes in Flagellin Glycosylation Affect *Campylobacter* Autoagglutination and Virulence. *Mol. Microbiol.* **2006**, *60*, 299-311.
35. Knirel, Y. A.; Rietschel, E. T.; Marre, R.; Zähringer, U., The Structure of the O-Specific Chain of *Legionella Pneumophila* Serogroup 1 Lipopolysaccharide. *Eur. J. Biochem.* **1994**, *221*, 239-245.
36. Kenne, L.; Lindberg, B.; Schweda, E.; Gustafsson, B.; Holme, T., Structural Studies of the O-Antigen from *Vibrio Cholerae* O:2. *Carbohydr. Res.* **1988**, *180*, 285-294.
37. Watson, D. C.; Wakarchuk, W. W.; Leclerc, S.; Schur, M. J.; Schoenhofen, I. C.; Young, N. M.; Gilbert, M., Sialyltransferases with Enhanced Legionaminic Acid Transferase Activity for the Preparation of Analogs of Sialoglycoconjugates. *Glycobiology* **2015**, *25*, 767-773.
38. Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q.; Chen, X., A Multifunctional *Pasteurella Multocida* Sialyltransferase: A Powerful Tool for the Synthesis of Sialoside Libraries. *J. Am. Chem. Soc.* **2005**, *127*, 17618-17619.
39. Watson, D. C.; Leclerc, S.; Wakarchuk, W. W.; Young, N. M., Enzymatic Synthesis and Properties of Glycoconjugates with Legionaminic Acid as a Replacement for Neuraminic Acid. *Glycobiology* **2010**, *21*, 99-108.
40. Huang, K.; Parmeggiani, F.; Ledru, H.; Hollingsworth, K.; Mas Pons, J.; Marchesi, A.; Both, P.; Matthey, A. P.; Pallister, E.; Bulmer, G. S.; Van Munster, J. M.; Turnbull, W. B.; Galan, M. C.; Flitsch, S. L., Enzymatic Synthesis of N-Acetylglucosamine from Lactose Enabled by Recombinant B1,4-Galactosyltransferases. *Org. Biomol. Chem.* **2019**, *17*, 5920-5924.
41. Sittel, I.; Galan, M. C., Chemo-Enzymatic Synthesis of Imidazolium-Tagged Sialyllactosamine Probes. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 4329-4332.
42. Galan, M. C.; Tran, A. T.; Bernard, C., Ionic-Liquid-Based Catch and Release Mass Spectroscopy Tags for Enzyme Monitoring. *Chem. Comm.* **2010**, *46*, 8968-8970.
43. Galan, M. C.; Tran, A. T.; Bromfield, K.; Rabbani, S.; Ernst, B., Ionic-Liquid-Based Ms Probes for the Chemo-Enzymatic Synthesis of Oligosaccharides. *Org. Biomol. Chem.* **2012**, *10*, 7091-7097.
44. Galan, M. C.; Jones, R. A.; Tran, A.-T., Recent Developments of Ionic Liquids in Oligosaccharide Synthesis: The Sweet Side of Ionic Liquids. *Carbohydr. Res.* **2013**, *375*, 35-46.
45. Tsukamoto, H.; Takakura, Y.; Mine, T.; Yamamoto, T., *Photobacterium* Sp. Jt-Ish-224 Produces Two Sialyltransferases, A-/B-Galactoside A2,3-Sialyltransferase and B-Galactoside A2,6-Sialyltransferase. *J. Biochem.* **2007**, *143*, 187-197.
46. Mine, T.; Katayama, S.; Kajiwara, H.; Tsunashima, M.; Tsukamoto, H.; Takakura, Y.; Yamamoto, T., An A2,6-Sialyltransferase Cloned from *Photobacterium Leigognathi* Strain Jt-Shiz-119 Shows Both Sialyltransferase and Neuraminidase Activity. *Glycobiology* **2009**, *20*, 158-165.
47. Schmölzer, K.; Czabany, T.; Luley-Goedl, C.; Pavkov-Keller, T.; Ribitsch, D.; Schwab, H.; Gruber, K.; Weber, H.; Nidetzky, B., Complete Switch from A-2,3- to A-2,6-Regioselectivity in *Pasteurella Dugmatis* B-D-Galactoside Sialyltransferase by Active-Site Redesign. *Chem. Comm.* **2015**, *51*, 3083-3086.
48. Ni, L.; Chokhawala, H. A.; Cao, H.; Henning, R.; Ng, L.; Huang, S.; Yu, H.; Chen, X.; Fisher, A. J., Crystal Structures of *Pasteurella Multocida* Sialyltransferase Complexes with Acceptor and Donor Analogues Reveal Substrate Binding Sites and Catalytic Mechanism. *Biochemistry* **2007**, *46*, 6288-6298.

49. Trott, O.; Olson, A. J., Autodock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* **2010**, *31*, 455-461.
50. L'vov, V. L.; Shashkov, A. S.; Dmitriev, B. A., Antigenic determinants of bacteria. The structure of the repeating unit of a specific polysaccharide from *Shigella boydii* type 7. *Bioorg. Khim.* **1987**, *13*, 223-233.
51. Staaf, M.; Weintraub, A.; Widmalm, G., Autodock Vina: Structure determination of the O-antigenic polysaccharide from the enteroinvasive *Eschericia coli* O136. *Eur. J. Biochem.* **1999**, *263*, 656-661.